The Exosporium Layer of Bacterial Spores: a Connection to the Environment and the Infected Host

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(continued)

SUMMARY

Much of what we know regarding bacterial spore structure and function has been learned from studies of the genetically well-characterized bacterium *Bacillus subtilis*. Molecular aspects of spore structure, assembly, and function are well defined. However, certain bacteria produce spores with an outer spore layer, the exosporium, which is not present on *B. subtilis* spores. Our understanding of the composition and biological functions of the exosporium layer is much more limited than that of other aspects of the spore. Because the bacterial spore surface is important for the spore's interactions with the environment, as well as being the site of interaction of the spore with the host's innate immune system in the case of spore-forming bacterial pathogens, the exosporium is worthy of continued investigation. Recent exosporium studies have focused largely on members of the *Bacillus cereus* family, principally *Bacillus anthracis* and *Bacillus cereus*. Our understanding of the composition of the exosporium, the pathway of its assembly, and its role in spore biology is now coming into sharper focus. This review expands on a 2007 review of spore surface layers which provided an excellent conceptual framework of exosporium structure and function (A. O. Henriques and C. P. Moran, Jr., Annu Rev Microbiol **61**:555–588, 2007, http://dx.doi.org/10.1146/annurev.micro.61.080706.093224). That review began a process of considering outer spore layers as an integrated, multilayered structure rather than simply regarding the outer spore components as independent parts.

INTRODUCTION

Many soil-dwelling bacteria of the phylum *Firmicutes* utilize sporulation as a survival mechanism whereby they cease vegetative growth and form an endospore inside the mother cell cytoplasm in times of unfavorable growth conditions, such as nutrient limitation. The spore is a survival rather than a replicative mechanism, as only a single spore develops within the cytoplasm of the sporulating cell (an endospore within a mother cell). The spore is released from the mother cell by lysis, and the mature spore exists as a dormant form of the cell that lacks metabolic activity (1, 2). Spores can persist in the environment for long periods.

Spore components include a core consisting of a cell with a dehydrated cytoplasm bearing high concentrations of calcium dipicolinate (Fig. 1). The core is enveloped in a cortex layer consisting of a modified peptidoglycan. External to the cortex is a proteinaceous shell referred to as the spore coat, often consisting of morphologically distinct inner and outer coat layers. The *Bacillus subtilis* coat comprises about 70 different proteins (3). Spores are highly refractive to environmental insults, such as desiccation, UV irradiation, heat, and exposure to organic solvents. When favorable growth conditions return, spores are stimulated to undergo a process of germination and outgrowth whereby the dormant spore takes up water, releases calcium ions, resumes
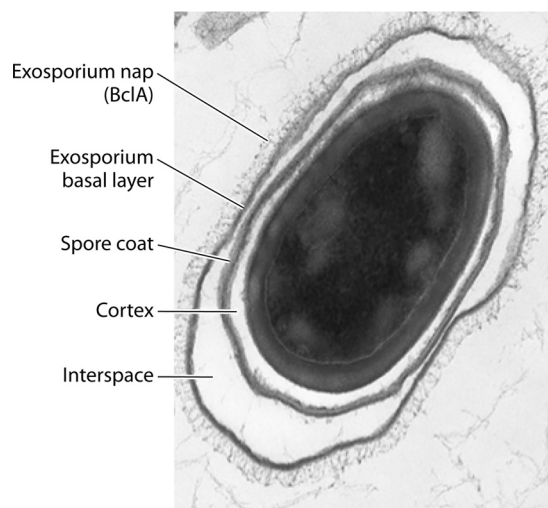
FIG 1 Transmission electron micrograph of a *B. anthracis* spore.

metabolic activity, and degrades the cortex and the vegetative cell emerges from the spore coat shell. Spore germination involves signaling from small-molecule germinants that diffuse across the outer spore layers and bind to receptors in the cytoplasmic membrane (4).

Endospore-producing bacteria have substantial impacts on human and animal health. Some are human and/or animal pathogens, whereas others affect humans more indirectly, such as insect pathogens or agents of food or product spoilage. *Bacillus anthracis* is an important zoonotic pathogen that causes anthrax, a fatal septicemia in ruminants. Human infections result in cutaneous, gastrointestinal, or pulmonary anthrax, depending on the site of spore entry into the patient. Gastrointestinal and pulmonary anthraces are associated with high mortality rates, and anthrax spores are a concern as a weapon of bioterrorism (5). *Bacillus cereus* is a common cause of food poisoning (6). It is also an invasive pathogen responsible for systemic infections, including endophthalmitis, bloodstream infections, endocarditis, and respiratory tract infections (7). Severe and lethal *B. cereus* infections have been reported for newborn infants, especially among those born prematurely (8, 9). *B. cereus* infections in newborns include involvement of the central nervous system or respiratory tract, primary bacteremia, and sepsis. *Bacillus thuringiensis*, a species closely related to *B. anthracis* and *B. cereus*, is an insect pathogen widely utilized in agriculture as a bioinsecticide (10). Sporulating cells of *B. thuringiensis* produce a crystal toxin that is poisonous to insects in the orders Lepidoptera, Coleoptera, and Diptera (11).

The anaerobic spore former *Clostridium difficile* is the causal agent of nosocomial antibiotic-associated diarrhea and pseudomembranous colitis and is an important emerging public health concern (12–14). Infection with *C. difficile* recently sur-

passed methicillin-resistant *Staphylococcus aureus* infection as the most common hospital-acquired infection in the United States (15). It is estimated that nosocomial *C. difficile* infections increase the cost of hospitalization 4-fold compared with matched cohorts, and these infections have been estimated to result in $4.8 billion in excess costs in U.S. acute-care facilities (12). Additionally, 20 to 27% of all *C. difficile* cases are community associated, with an incidence of 20 to 30 per 100,000 population (16). *Clostridium perfringens* is one of the most common causes of human food-borne illness, with an estimated million cases of foodborne illness each year in the United States (17, 18). *C. perfringens* also causes infections of skin and soft tissue, gastroenteritis, gas gangrene, necrotizing enteritis, liver abscess, bacteremia, endophthalmitis, and septic shock with acute hemolysis (19–21). Clostridia are also the second most common cause of anaerobic bacteremia, and the 30-day mortality rate for infected patients is 27 to 44% (19).

Botulism results from exposure to a potent neurotoxin produced by the spore-forming bacterium *Clostridium botulinum* (22–24). There are five main kinds of botulism. Foodborne botulism is caused by eating foods that contain the botulinum toxin. Canned (especially home-canned) and vacuum-packed foods, which provide the anaerobic environment necessary for contaminating spores to germinate and the vegetative cells to produce the toxin, are the foods usually implicated. Wound botulism is caused by wound contamination with *C. botulinum* spores, and currently the majority of these cases are associated with black-tar heroin injection. Infant botulism is caused by consumption of the spores of the botulinum bacteria, which then grow in the intestines and release toxin in children under the age of 1 year. Adult intestinal toxemia (adult intestinal colonization) botulism is a very rare form of botulism that occurs among adults by the same route as infant botulism. Iatrogenic botulism can occur from accidental overdose of botulinum toxin in medical or cosmetic product formulations. In the United States, an average of 145 cases of botulism are reported each year, with approximately 15% being foodborne, 65% being infant botulism, and 20% being wound associated (22).

The spore-forming process of *B. subtilis* is the best studied of such processes (25). Early in sporulation, the cell begins to divide asymmetrically, and the division septum undergoes remodeling, resulting in engulfment of the smaller cell compartment. This results in the formation of a smaller, double-membrane-bound forespore compartment within the mother cell. During spore maturation, the mother cell directs the synthesis and assembly of the outermost spore layers, including spore coat proteins and, depending on the bacterial species, additional outer layers, including the exosporium.

THE EXOSPORIUM LAYER OF BACTERIAL SPORES

> **Under higher magnification, every spore appears to be of egg-like shape and embedded within a ball-shaped glass-like mass looking like a light, narrow ring surrounding the spores.**
> —Robert Koch (26)

In his classic 1876 paper on the etiology of anthrax, Robert Koch first described what is now known as the exosporium layer of the *Bacillus anthracis* spore (26). The term "exosporium" was later coined by Flügge in 1886 (27). Bacterial endospores can be placed
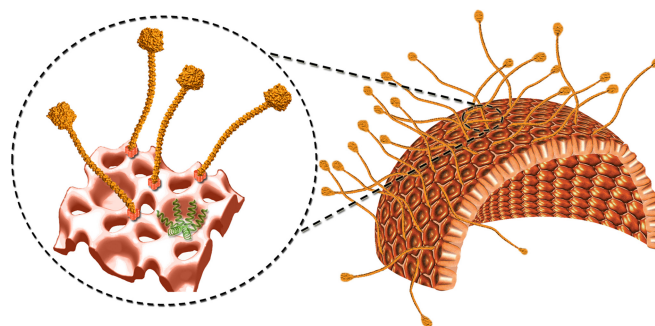

FIG 2 Schematic diagram illustrating a model for the exosporium of the *B. cereus* family. The exosporium basal layer consists of a two-dimensional lattice of cups opening out to the environment on the convex outer face of the layer. There are channels penetrating the basal layer. The collagen-like fibrils of the BclA nap are shown schematically attached to the convex face, terminating in the globular tumor necrosis factor-like C-terminal domain. (Reprinted from reference 38 with permission of the publisher.)

into two categories: those containing a distinct exosporium and those lacking this structure. The best studied of the former group are those of *B. anthracis* and *B. cereus*. *Bacillus subtilis* spores are the best-studied example of the exosporium-less category. Much of what we know regarding spore assembly has been learned from genetic and biochemical studies with *B. subtilis*. Consequently, there is a wealth of knowledge regarding initiation of sporulation through spore coat assembly. Because *B. subtilis* lacks an exosporium, information regarding exosporial composition and its assembly process is considerably more limited.

The exosporium of the *B. cereus* family of bacteria consists of a basal layer surrounded by an external nap of hairlike projections (28–30) (Fig. 1). The collagen-like glycoprotein BclA (*Bacillus* collagen-like protein A) is the principal component of this hairlike nap and is an immunodominant spore antigen (31–33). The exosporium basal layer is a shell composed of a number of different proteins (33–35).

Advances in electron microscopic imaging techniques have led to spectacular improvements in our understanding of the exosporium architecture of spores from the *B. cereus* family (36–39). The basic structures of the exosporium are similar for *B. anthracis*, *B. cereus*, and *B. thuringiensis*. The balloon-like exosporium is deformable and irregularly shaped and possesses numerous folds and creases. The exosporium is separated from the spore coat by extensive distances (for example, 500 nm in *B. anthracis* [39]) yet lies in close proximity to the spore coat at other sites around the spore. The region between the basal layer of the exosporium and the outer spore coat is referred to as the interspace (35). The basal layer of the *B. anthracis* exosporium is approximately 12 to 16 nm thick and appears to be comprised of two, approximately 5-nm-thick sublayers (39). The basal layer has a crystalline structural organization with a 6-fold symmetry and a periodic spacing of 7 nm. The external face of this structure consists of a series of hexagonally packed concave cups in a honeycomb pattern, with the open ends oriented toward the external environment (Fig. 2) (38). The spore core-facing side of this structure consists of a honeycomb pattern of hexameric crowns with a diameter of ~7.5 to 9 nm (Fig. 2). The open ends of the crowns abut one another to form channels between the two faces of the basal layer. The channels have a diameter of ~20 to 34 Å (36, 38). A channel of this size is
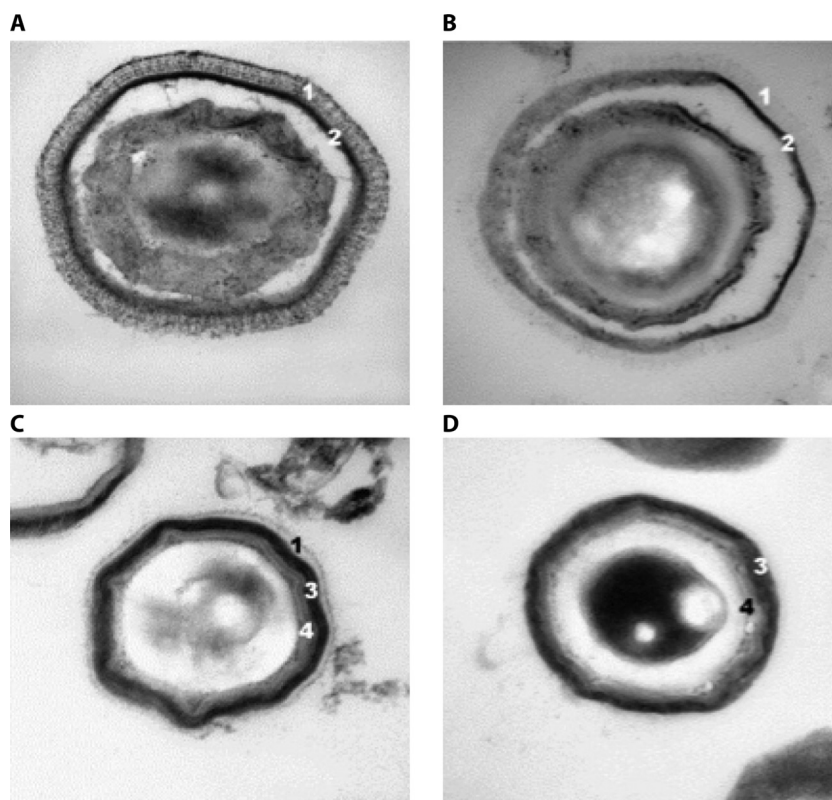
FIG 3 Electron micrographs of spores from sporulated cultures. (A) *B. anthracis* stained with ruthenium red. 1, glycoprotein nap; 2, basal membrane. (B) *B. anthracis* without ruthenium red staining. 1, glycoprotein nap; 2, basal membrane. (C) *B. subtilis* stained with ruthenium red. 1, glycoprotein nap; 3, outer coat; 4, inner coat. (D) *B. subtilis* without ruthenium red staining. 3, outer coat; 4, inner coat. (Reprinted from reference 48 with permission of the publisher [copyright Elsevier 2004].)

sufficiently large to permit entry of small-molecule germinants, such as alanine or inosine, but is too small to permit diffusion of larger molecules, such as proteins. The measured channel size is consistent with the results of spore permeation studies (40). This channel arrangement confers the semipermeable barrier properties of the exosporium.

The BclA nap filaments of the *B. anthracis* spore span 14 to 70 nm (32, 39) and are attached to the outer surface of the basal layer. The length of the filaments is a function of the number of collagen-like triplet amino acid repeats in the BclA protein (32). The hairlike nap covers the entire surface of the exosporium, with an interfilament spacing of approximately 7 nm, the same spacing periodicity as the basal layer crystalline array. The trimeric BclA filaments are oriented with their N termini at the basal layer and their C-terminal domains appearing as a 6-nm knob at the end of the outward-extending filaments.

The exosporium is anchored to the spore coat layer via a number of protein-protein interactions. These have been identified through the isolation of mutants that fail to stably attach the exosporium and from which, as a consequence, the exosporium is released as sheets separate from the mature spores upon lysis of the mother cells. Proteins implicated in exosporium attachment include the spore coat protein CotE and the exosporium- or interspace-localized proteins CotY, ExsA, ExsB, ExsY, and ExsM (1, 41–45). Whether these proteins participate directly in exosporium attachment or indirectly, through proper positioning of proteins actually involved in the attachment, has not been established.

CotE is likely to be the spore coat-associated part of the connector between the spore coat and the exosporium. ExsA and/or ExsB may also be part of this connection chain.

THE SPORE CRUST LAYER

B. subtilis lacks an obvious exosporium layer and has the outer spore coat layer as its surface. This is consistent with the finding that known *B. cereus* family exosporium genes are not present in the *B. subtilis* genome. However, the surfaces of *B. subtilis* spores may not be that dissimilar from those of *B. cereus* family spores. Treatment of *B. subtilis* spores with urea and mercaptoethanol resulted in the detachment of a layer from the underlying outer coat material that resembled the basal membrane of the exosporium of *B. anthracis* (46, 47). Use of a ruthenium red staining technique which has been used for visualization of carbohydrate capsules by electron microscopy resulted in the visualization of filaments on the surfaces of *B. subtilis* spores (Fig. 3) (48). Spore surface filaments are present and are possibly glycoprotein in nature, although this conclusion is drawn with caution, as ruthenium red staining is not totally glycoprotein specific. Spore surface-specific carbohydrates exist in *B. subtilis* (49). These carbohydrates display structural similarity to those of the *B. cereus* family spores (49, 50). Production and/or attachment of polysaccharides to the *B. subtilis* spore surface was shown to be *spsM* dependent (51). This determinant is the site of SPβ prophage integration, and functional SpsM is produced only following excision of the prophage during sporulation (51). Spores from *spsM*-

negative mutants had altered surface properties, including increased adherence to glass and enhanced aggregation in aqueous environments. The presence of spore surface glycoprotein filaments raises the possibility that functions contributed by the BclA nap on the *B. cereus* family spores are provided by other surface glycoproteins in certain exosporium-less spores.

Additional support for the presence of *B. subtilis* spore surface glycoproteins came from a study of the *cge* locus. Mutations in the *cgeAB* and *cgeCDE* operons of *B. subtilis* resulted in spores with altered surface properties (spores tended to clump, pelleted more tightly, and adhered more strongly to plastic and glass surfaces), and it was speculated that this was due to changes in glycosylation of surface proteins (52). In an analysis of spore coat proteins involving fluorescent protein fusions, the *B. subtilis* spore coat was predicted to be comprised of four distinct layers, including an outermost glycoprotein layer, named the "crust," that is separated from the outer coat by a small space (53). Genetic and localization studies have shown that *cgeA* and genes in the *cotVWXYZ* locus are involved in spore crust formation (53, 54). Interestingly, the CotZ protein, which plays a role in the assembly of the crust layer of *B. subtilis*, displays sequence similarity to the ExsY protein (35% identity and 46% similarity over 141 residues), a protein involved in exosporium assembly in *B. anthracis* (42). *B. subtilis* CotE, CotV, CotY, and CotW coat proteins expressed in *Escherichia coli* can self-assemble intracellularly into stable complexes (55). These proteins form one-dimensional fibers, two-dimensional sheets, and three-dimensional stacks. With CotY, the mode of assembly resembles that of the exosporium basal layer described for *B. anthracis* and *B. cereus* (55). CotY is a component of the *B. subtilis* crust layer and the *B. cereus* group exosporium layer. The propensity of these Cot proteins to self-assemble may contribute to the spore coat, crust layer, or exosporium assembly processes.

The questions of whether the crust is functionally equivalent to the surface of the exosporium and whether crust-like layers are found on the outer spore coat in exosporium-containing spores remain unanswered.

THE BOTTLE CAP MODEL OF EXOSPORIUM STRUCTURE

In a study of an *exsY* mutant of *B. anthracis*, Turnbough and coworkers discovered that the exosporium layer is not a uniform structure but is comprised of two distinct entities of differing compositions (42, 56). The "bottle cap" part of the exosporium is synthesized first, originating at the mother cell central pole of the spore. After assembly of the bottle cap portion of the exosporium reaches the cap-noncap junction, the noncap portion (~75%) of the exosporium is assembled (Fig. 4). The noncap portion fails to assemble in an *exsY*-null mutant, yielding spores with a loosely attached, cap-only exosporium that is readily lost when the spore is released from the mother cell (42). The protein compositions of the cap and noncap regions of the exosporium differ. CotY is a cap-specific protein, whereas BclB, ExsY, and Alr are found in the noncap portion of the exosporium (56–58). BclA is found on the exosporium surface in both cap and noncap sites, whereas BxpB is found in larger amounts, but not exclusively, in the noncap portion of the exosporium (58). The junction between the cap and noncap regions may play an important role in exosporium-containing spores during the germination process. Steichen et al. (56) demonstrated that during spore germination and outgrowth, the emerging vegetative cell escapes from its exosporium shell through the displaced cap, suggesting that the cap is designed to
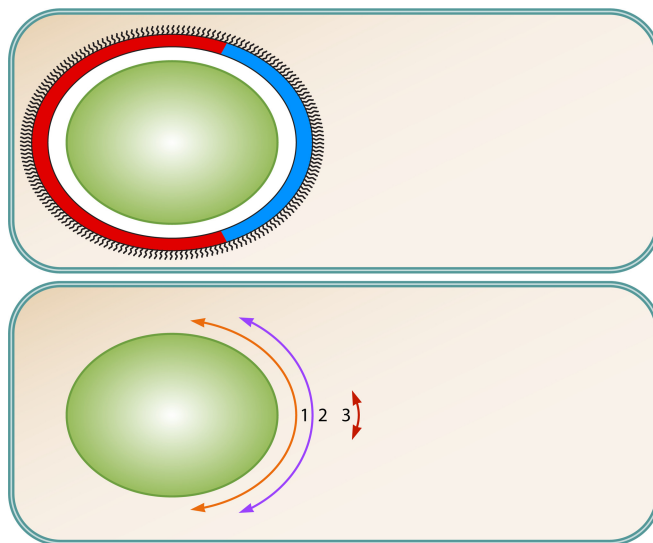
FIG 4 (Top) Illustration of a mature spore in a sporulating cell of *B. anthracis*. The bottle cap portion of the exosporium, at the mother cell central pole of the spore, is depicted in blue, and the noncap exosporium is shown in red. (Bottom) Three distinct exosporium synthesis events which originate at the mother cell central pole of the spore. (1) The first event is the exosporium scaffold layer portion of the basal layer synthesis involving CotY and ExsY. (2) This is followed closely by outer basal layer assembly, which includes BxpB and the BclA nap layer. When the BxpB layer is then approximately 75% complete, cleavage of the BclA N terminus after amino acid 19 (arrow 3) begins at the mother cell central spore pole, and this wave of cleavage follows the assembly around the spore periphery (57).

pop off the exosporium to permit the emergence of the outgrowing cell. Mutants of *B. anthracis* lacking the BclB collagen-like protein have an exosporium that exhibits damage and loss of the exosporium at one pole (59). Furthermore, the distribution of exosporium proteins, such as CotY and BxpB, is altered in this mutant, suggesting that BclB is required for correct exosporium assembly. The normally cap-associated CotY protein is distributed more completely around the spore, and BxpB is largely restricted to the noncap pole in *bclB*-null spores. Localization studies with the BclB-negative spores suggested that without BclB, the cap encompasses >75% of the exosporium's circumference, instead of the usual ~25%. BclB, a non-cap-region exosporium protein, may participate in the transition in exosporium composition that must take place as the assembly of the cap stops and the noncap assembly begins during the discontinuous assembly of the exosporium (56, 58). It has been postulated that in a BclB-null strain, the transition zone between the cap and noncap sections of the exosporium is either displaced to the opposite pole of the spore or enlarged to encompass the midsection of the spore (58).

ASSEMBLY OF THE EXOSPORIUM

Genetic studies on spore assembly in *B. subtilis* have ordered the steps in spore production based on the effects of particular mutations on downstream processes (60). Analysis of mutants involving exosporium genes of *B. anthracis* and *B. cereus* are more limited but do shed some light on the overall exosporium biosynthetic process. The *B. cereus* exosporium first appears in the sporulating mother cell as a small lamellar structure adjacent to but distinct from the outer forespore membrane (61). Electron microscopy and protein localization studies have shown that synthe-

sis of the exosporium initiates at the mother cell central spore pole, the region of the exosporium that will become the bottle cap (35, 56, 57, 62). Exosporium assembly progresses by continued deposition of proteins at the leading edge of this nascent exosporium, terminating at the opposite spore pole. However, there is a transition in the incorporation of proteins as synthesis reaches the end of the cap region (approximately 25% of the spore circumference), as the composition of the noncap region of the exosporium differs from that of the bottle cap region. As discussed above, there are proteins involved in anchoring the exosporium to the spore coat. The exact nature of the spore coat anchor has yet to be defined. However, the spore coat protein CotE is involved in this process (43). In CotE-negative spores, the exosporium assembles in sheets which are not anchored to the spore, and mature spores have only fragments of exosporium associated with them. However, the exosporium produced in this mutant possesses the BclA nap layer, a late addition in the exosporium assembly process. Thus, anchoring of the exosporium to the underlying spore coat layer is not required for the exosporium assembly process to proceed.

Known exosporium basal layer proteins include ExsY, CotY, BxpB (also known as ExsFA), and ExsFB. The former two proteins are involved in an earlier stage of exosporium synthesis than the latter pair. Loss of ExsY in *B. anthracis* results in the production of only the cap region of the exosporium, with no noncap region of the exosporium evident in either late-stage sporulating cells or released mature spores (42). The exosporium cap region is not firmly anchored to the spore and is lost, presumably due to fluid shear forces, when spores are prepared in a liquid medium (42, 44). *B. cereus* mutants devoid of both ExsY and CotY lack an exosporium layer (42, 44), whereas loss of ExsY results in spores with only fragments of exosporium-like material adherent to the spores. The absence of exosporium caps on *B. cereus exsY* mutant spores was likely the result of shear forces in place during preparation of the spores (44). *B. cereus* single *cotY* mutants produce an intact exosporium. With the *B. anthracis exsY* mutant, no fragments of apparent exosporium remain attached to the released spore as observed with *B. cereus*. ExsY appears to be essential for noncap exosporium assembly, whereas its paralog CotY is not required for production of an intact exosporium (44). The cap region exosporium produced in the *B. anthracis exsY* mutant possesses the BclA nap layer (42). Since BclA incorporation occurs later in the exosporium biosynthesis process, this indicates that later-stage assembly events occur in the cap independently of production of the noncap region.

BxpB (ExsFA) is an exosporium basal layer protein that, along with its paralog ExsFB, is required for assembly of the BclA nap layer. Loss of both BxpB and ExsFB is required for complete loss of BclA. Without BxpB, only about 25% of BclA is incorporated onto the spore surface. Loss of ExsFB results in only a very modest reduction in BclA spore levels (63, 64). Mutants lacking both BxpB and ExsFA produce an intact exosporium encircling the spore. However, this structure is fragile, is easily damaged, and is lost from the spores over time (63). This observation suggests that BxpB and ExsFB are assembled into the growing exosporium at a later stage than that involving ExsY and CotY, since loss of the latter pair results in no visible exosporium structure. Thus, the exosporium is likely assembled in two or more distinct layers, consistent with the two layers of exosporium basal layer evident by electron microscopy, as described above. CotY (at the cap) and

ExsY (at the noncap region) are incorporated into an early scaffold layer (the sac sublayer of Henriques and Moran [1]). Subsequently, BxpB and ExsFB are added in a layer that is likely to be positioned externally relative to the ExsY/CotY-containing scaffold layer (Fig. 4).

Much of what is known regarding exosporium synthesis concerns incorporation of the nap glycoprotein BclA and the basal layer protein BxpB into the developing exosporium of *B. anthracis*. Both proteins appear in the mother cell cytoplasm approximately 4 to 5 h into sporulation (in broth cultures shaken at 37°C). The majority of each of these proteins first appears in the form of higher-molecular-weight complexes rather than monomers, indicating that stable complex formation occurs in the mother cell cytoplasm well in advance of placement of these proteins at the site of the developing exosporium. Appearance of BclA and BxpB in the sporulating cell initiates an hour prior to evidence of initial assembly on the developing spore (57). The BxpB-containing and BclA-containing complexes observed in Western blots are similar in size, suggesting that interactions between these two proteins may occur. The protein-protein interactions in these complexes are stable to boiling in the presence of 1% SDS and 8 M urea and in the presence of reducing agents.

An N-terminal targeting sequence of BclA and certain other collagen-like proteins is responsible for positioning these proteins at the site of the developing exosporium (57, 58, 65–67). No such localization domain is present in the BxpB sequence. Therefore, my colleagues and I have proposed a model whereby BxpB is delivered to the spore by virtue of forming a complex with BclA and possibly, to a lesser extent, with other exosporium-targeting motif sequences (58). In keeping with this, we observed a reduction in the amount of BxpB extractable from spores of a BclA-negative strain, a finding similar to that observed by others (65, 66, 68). However, this model is disputed by Rodenburg et al. (39), based on their analysis of *B. anthracis* spore structure by high-resolution electron microscopy. They propose that loss of BclA has no effect on BxpB spore incorporation. The assignment of these structures as BxpB, however, was not validated either biochemically or immunologically, so the data remain speculative. How their results can be reconciled with the reduction in BxpB levels in spores in which BclA failed to localize, as previously reported by Tan and Turnbough (65), was not addressed in their more recent report.

Exosporium assembly initiates at the mother cell central pole of the spore (the cap region of the exosporium). Assembly begins with the incorporation of an as yet undefined set of proteins (but likely including CotY and ExsY) which provides the scaffold for assembly of the basal layer. After scaffold assembly has initiated, the assembly of the BclA- and BxpB-containing complexes occurs at this mother cell central spore pole and follows scaffold assembly around the spore (57, 66). When positioning of BclA-containing complexes has occurred over approximately 75% of the spore, proteolytic cleavage of the BclA N terminus after amino acid 19 occurs, beginning at the cap end of the exosporium, and the N-terminal peptide is released into the mother cell cytoplasm (57). This cleavage event may be associated with covalent attachment of BclA to an exosporium basal layer protein, thus anchoring the nap glycoprotein to the exosporium surface. Late in the assembly process, after BclA has been incorporated onto the spore surface, glycosylation of BclA occurs (66).
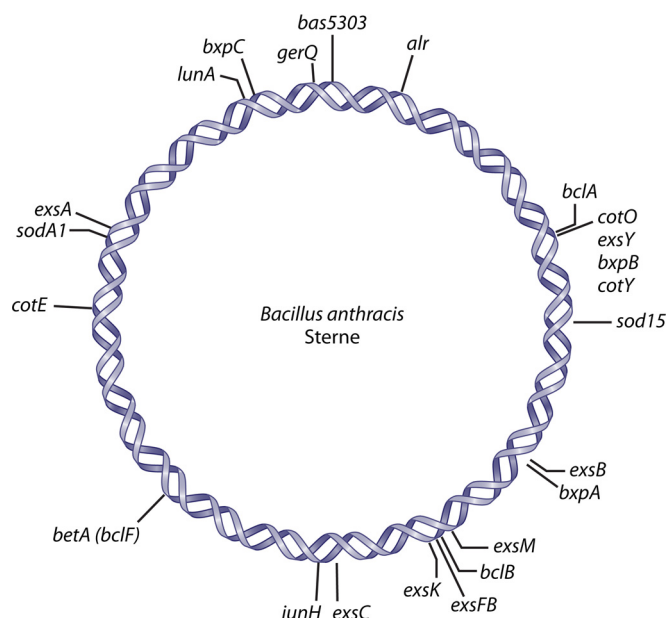
FIG 5 *B. anthracis* Sterne chromosome with map positions of exosporium determinants.

BACILLUS EXOSPORIUM PROTEINS

Exosporium proteins have principally been defined for strains of *B. anthracis* and *B. cereus*. Except where noted below, exosporium proteins of the *B. cereus* family (*B. anthracis*, *B. cereus*, and *B. thuringiensis*), other than collagen-like proteins, tend not to be found, at least as conserved homologs, in other species of *Bacillus* or in *Clostridia*. Therefore, an absence of these proteins in genomic sequences does not rule out the presence of an exosporium. The exosporium-associated Cot proteins display sequence similarity with spore coat proteins of other spore formers, and their locations within spores in the non-*B. cereus*-family spore-forming bacteria have not been defined. The chromosomal locations of putative exosporium determinants in *B. anthracis* are shown in Fig. 5. The determinants map to multiple sites in the genome rather than being clustered in a single or small number of loci. The proteins indicated below have been shown to be either exosporium associated or possibly so, in cases where the actual location has not been determined precisely. The BAS numbers indicate the Sterne strain coding sequence (CDS) numbers (NCBI accession numbers NC_005945.1 and AE017225.1).

Alr (BAS0238)

Alanine racemase is an enzyme that can convert L-alanine (a germinant for *B. anthracis*) to D-alanine (an inhibitor of germination) (56, 69). The presence of Alr in the spore may function to inhibit premature germination of the spore under suboptimal growth conditions, such as in the intracellular macrophage environment. It may also prevent premature germination during sporulation (69, 70). The distribution of Alr in the exosporium of *B. anthracis* is not uniform. The enzyme is found only in the noncap region of the spore, which comprises ~75% of the spore and excludes the pole at which exosporium synthesis initiates (69).

Arginase

B. anthracis endospores exhibit arginase activity (71). This activity is reduced when the exosporium is removed by sonication and retained in exosporium preparations (71). The spore-associated arginase possibly competes with host cell nitric oxide synthase (NOS 2) for its L-arginine substrate (72). As macrophage-generated nitric oxide is important for microbial killing, the presence of arginase in internalized *B. anthracis* spores may function to decrease intracellular arginine levels and thus reduce the concentration of this substrate for host NOS 2. This may enhance the bacterium's ability to survive following germination in this intracellular compartment during the initial stages of infection. The arginase enzyme has not yet been localized to a specific site within the exosporium or interspace layer of the spore. There are two proteins annotated as arginases encoded in the *B. anthracis* Sterne genome (BAS0155 and BAS2260). Of these, only BAS0155 has an expression profile consistent with a potential spore-associated protein (73), and the open reading frame is preceded by a putative σ^K promoter sequence.

BAS5303

The BAS5303 protein was identified as an exosporium component in a proteomic study of the *B. anthracis* spore (74). Inclusion of this 133-residue protein of unknown function enhanced the efficacy of an anti-protective antigen (anti-PA) vaccine in a mouse model of infection, and anti-BAS5303 antibodies were found to increase phagocytic uptake of spores (68). Although the location of this protein has not been mapped precisely, it was reported to likely lie below the BclA nap layer on the exosporium, based on increased exposure to antibodies in the absence of BclA (68). The *bas5303* determinant is monocistronic and expressed at a very late stage of sporulation (73). The open reading frame is preceded by two putative σ^K promoter elements, consistent with this expression profile.

BclA (BAS1130)

BclA was the first spore surface glycoprotein discovered in *B. anthracis* and is the prominent protein component of the exosporium nap layer (31, 33). The filaments of the nap are composed of this trimeric collagen-like protein. BclA is comprised of three domains. The N-terminal domain (NTD) contains the targeting and attachment domains whereby the protein is apparently covalently attached to the basal layer of the exosporium (57, 65, 66, 75). Stable attachment requires proteolytic cleavage of the NTD between residues serine 19 and alanine 20. The NTD possesses a targeting sequence which is required for the positioning of BclA around the developing exosporium and the attachment domain containing the proteolytic cleavage site (Fig. 6) (31, 57, 65, 66). The targeting sequence is conserved in a subset of other exosporium-associated collagen-like proteins, whereas the attachment domain, which lies upstream of the targeting sequence, is not identifiable in these proteins (Fig. 6) (57).

The NTD is followed by a collagen repeat region consisting of GX_1X_2 (primarily GPT) triplet amino acid repeats. These repeats are the primary attachment point for rhamnose-oligosaccharides within BclA (76). The number of glycine-containing triplet amino acid repeats in BclA varies among strains (17 to 91 triplet repeats), and this variation is responsible for the different lengths of the hairlike nap observed on spores of different *B. anthracis* strains (32, 33). The 134-residue carboxy-terminal domain is organized

BclA	M S N N N Y S N G L N P D E S L S A S A F D P N L V G P T L P P I P P
BclB	M K Q N D K L W L D K G I I G P E N I G P T F P V L P P
BclE	M V K V V E G N G G K S K I K S P L N S N F K I L S D L V G P T F P P V P T
BetA/BclF	M S E K Y I I L H G T A L E P N L I G P T L P P I P P

FIG 6 N-terminal amino acid sequences of *B. anthracis* collagen-like proteins possessing the exosporium assembly domain. The proteolytic cleavage site of BclA is denoted by an arrow. Attachment motif sequences are shown in red, and localization motif sequences are shown in green.

into an all-β structure with a jelly roll topology (77, 78). It is structurally similar (but dissimilar in sequence) to the globular domain of the human C1q complement protein, which belongs to the tumor necrosis factor-like family of proteins. The trimeric globular head formed by the C-terminal domain is thought to be the nucleation site for the formation of the collagen-like triple helix, as in several mammalian collagens (77).

The polysaccharide composition of BclA includes L-rhamnose, *N*-acetylgalactosamine, 3-*O*-methyl rhamnose, and anthrose [2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxyglucose; found at the nonreducing terminus of a tetrasaccharide] (50, 76). Daubenspeck et al. (76) identified a 715-Da tetrasaccharide [2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose] and a 324-Da disaccharide attached to BclA, likely through *N*-acetylgalactosamine residues at the reducing ends, making the polysaccharides actually pentasaccharides and trisaccharides. Multiple copies of the likely pentasaccharides are O-linked to the collagen-like region of BclA, apparently through threonine residues present in the many GXX repeats in this region. The attachment site of the trisaccharides was predicted to be outside the collagen-like repeat region. The BclA glycoprotein is present in strains of *B. cereus* and *B. thuringiensis*, but the presence of anthrose appears to be specific only to BclA from *B. anthracis* (76). However, antisera raised against anthrose cross-reacted with spores of certain *B. cereus* strains (79). The C-terminal domain of BclA from *B. cereus* ATCC 14579 was reported to be glycosylated, with 2-*O*-methyl-rhamnose and 2,4-*O*-methyl-rhamnose substitutions found in the C-terminal domain (80).

BclA has also been shown to be an immunodominant protein on the *B. anthracis* spore surface and contributes to the overall spore hydrophobicity (33, 81). This immunogenicity provides a potential benefit for anthrax vaccine development, as immunization with protective antigen (PA) and BclA protected mice and

guinea pigs from challenge with *B. anthracis* Sterne (pXO1$^+$/pXO2$^-$) or Ames strains better than immunization with PA alone (68, 81–83). Loss of BclA has little, if any, effect on sporulation rates of *B. anthracis*. However, germination of BclA-null spores was reported to occur more rapidly than that of wild-type spores *in vitro* (84).

The BclA protein has a predicted molecular weight (MW) of 37,000, but it first appears as higher-molecular-weight forms in sporulating cells (57, 66, 67). BclA extracted from spores is found in a heterogeneous band of complexes centered with an apparent MW of >250,000. The higher-molecular-weight forms are likely the result of trimerization of this collagen-like protein, its glycosylation, and its capacity to form covalent and noncovalent but SDS- and urea-resistant complexes with other exosporium proteins.

The *bclA* determinant (*bas1130*) is carried within a cluster of genes (*bas1127* to *bas1145*) which is organized as shown in Fig. 7. It possesses a putative promoter element recognized by the σ^K-bearing form of RNA polymerase, resulting in its expression approximately 3 h into sporulation in the mother cell compartment of the sporulating cell. The *bclA* determinant is flanked by genes predicted to be involved in glycosylation of this, and perhaps other, spore proteins. The *rmlABCD* genes are predicted to encode enzymes for rhamnose production, and inactivation of the *rmlA* determinant resulted in a loss of rhamnose incorporation into spores (85). *rmlA* mutant spores exhibited decreased binding to macrophages, suggesting a role for the carbohydrate moiety of the BclA glycoprotein in host cell interactions (85). Genes known to be associated with glycosylation of BclA are not found at a single locus on the *B. anthracis* genome. The anthrose component of the BclA glycoprotein is synthesized by the products encoded within a four-gene operon (*bas3322* to *bas3319*) not linked to *bclA* (86–88). Furthermore, the *bas5304* determinant was shown to be the epimerase required to produce *N*-acetylgalactosamine for BclA oligosaccharide biosynthesis (86).

FIG 7 *bclA* locus of the *B. anthracis* Sterne chromosome. Exosporium determinants are shown in black, rhamnose biosynthetic genes important for glycosylation of BclA are shown in light blue, the gene for the spore coat protein CotO is shown in dark blue, putative exosporium determinants are shown in brown, and genes encoding proteins which may be involved in glycosylation of BclA, and perhaps other spore collagen-like proteins, are shown in red (putative methyltransferases) and purple (putative glycosyltransferases).

BclB (BAS2281)

The second *B. anthracis* spore collagen-like glycoprotein to be identified, BclB, contains a GXT tandem repeat domain (89). 3-*O*-Methyl rhamnose, galactosamine, and rhamnose were found to be components of this glycoprotein. The BclB protein has sequence similarities to the ExsH and ExsJ proteins of *B. cereus* and *B. thuringiensis*. The BclB amino acid sequence at the N terminus is more divergent between *B. anthracis* and the ExsH/J proteins of the other two members of the *B. cereus* family. BclB possesses an N-terminal exosporium targeting motif similar to that of BclA, although no site corresponding to the N-terminal proteolytic cleavage site of BclA is found in the BclB amino acid sequence (57). BclB was determined to be present in the exosporium of *B. anthracis* spores by immunogold transmission electron microscopy (59). Immunofluorescence and flow cytometry studies indicated that BclB is surface exposed on spores, but a higher reactivity in spores lacking the BclA nap suggests that BclB is found principally beneath the BclA spore nap layer. The distribution of BclB around spores is not uniform, with this glycoprotein found in the 75% of the spore corresponding to the noncap region (58). BclB assembly into spores is prevented in *bxpB*-null mutant spores, suggesting that the BxpB basal layer protein is directly or indirectly required for BclB assembly. Spores from a Δ*bclB* mutant strain exhibited ruptures of the exosporium layer, usually at one pole of the elliptical spore (59). As a consequence of this exosporium instability, a population of exosporium-free spores is present in a sample of *bclB*-null spores. Loss of BclB also reduced the volume of the interspace region of the spores (58).

An absence of the BclB protein was found to have a pronounced effect on the distribution of other exosporium proteins (58). The normally cap-associated CotY protein was distributed more completely around the spore. BxpB is found in greater quantities in the noncap region of the exosporium than in the cap, but in the absence of BclB, the distribution of BxpB was largely restricted to the noncap pole.

BetA (BclF; BAS3290)

BetA (also referred to as BclF) is a collagen-like protein with the same overall architecture as that of BclA and BclB, with an N-terminal exosporium targeting motif followed by a collagen-like repeat sequence and then a C-terminal domain (Fig. 6) (90, 91). Expressed from an apparent σ^K promoter, its transcript level peaks later than those of *bclA* and *bclB* (73). BetA is found only in the *B. cereus* family of bacteria and possesses a small collagen-like repeat region which varies in length in different strains, from 20 repeats in *B. thuringiensis* Al Hakam to 11 repeats in *B. anthracis* Sterne and *Bacillus mycoides*. The differences in the collagen-like repeat region sizes among strains are primarily in the number of GIGITGPTGVTGXT motif repeats (1 motif in *B. anthracis* Sterne and Ames and 5 motif repeats in *B. thuringiensis* Al Hakam and *B. cereus* ATCC 14579). BetA resides in the exosporium basal layer, likely underneath BclA. BetA assembles at the spore surface at around hour 5 of sporulation, and its assembly is BxpB dependent, a feature common to the spore collagen-like glycoproteins with the N-terminal exosporium targeting motif (57, 58, 91). A *Bacillus megaterium* protein (for example, the sequence under GenBank accession number WP_047934722) displays 28% identity and 51% similarity over a 139-residue sequence with the *B. anthracis* BetA protein. Whether this protein is associated with the spore or exosporium in this species has not been determined.

BxpA (BAS2008)

BxpA is a glutamine- and proline-rich protein first identified in a proteomic analysis of the *B. anthracis* exosporium (34). BxpA contains repeated amino acid sequences of 5, 40, and 14 amino acids, and the three longest repeats are in tandem (33). BxpA is found in members of the *B. cereus* family, although sequence variation, including internal truncations, exists. The protein found in mature spores may be proteolytically processed, with only the C-terminal portion being spore associated (33, 92). Immunogold labeling suggested that most of the BxpA in the spore resides in the cortex layer, with no detectable amounts present in the exosporium (92). The YabG and Tgl proteases appear to be involved in BxpA processing during spore assembly, and the proteolytic cleavage may be associated with protein-cross-linking events (92).

BxpB (ExsFA; BAS1144)

The BxpB (also known as ExsFA) protein of the *B. cereus* family is an exosporium basal layer protein (63, 64). Spores from mutants lacking BxpB show a substantial reduction in BclA content, and the residual BclA is found predominantly at the cap region of the exosporium (35). Complete loss of BclA requires an additional mutation to inactivate ExsFB, an identically sized protein that exhibits 78% sequence identity with BxpB. Loss of both of the ExsF proteins results in the production of a structurally unstable exosporium layer which is readily lost from the spores (63). BxpB is synthesized in the mother cell compartment of sporulating cells and is immediately found in higher-molecular-weight complexes that are refractive to disruption by boiling in SDS in the presence of a reducing agent. BxpB is important, either directly or indirectly, for the incorporation of a number of exosporium proteins into the spore, including BclA, BclB, and BetA (57–59, 65, 66, 91). It is generally believed that the proteolytic processing of the N terminus of BclA results in covalent attachment of BclA to BxpB, although this has not been demonstrated definitively. However, the N-terminal domains of BclA and BxpB are sufficiently close in sporulating cells that fluorescent fusion proteins are capable of fluorescence resonance energy transfer (FRET) (66). Mutant spores of *B. anthracis* lacking BxpB were reported to have a defect in an early step of germination (35). In contrast, Steichen et al. (64) found that BxpB-negative spores displayed faster germination and outgrowth kinetics.

BxpC (BAS5053)

BxpC was identified as a 15.3-kDa putative exosporium protein of *B. anthracis* by Steichen et al. (33). BxpC has 40% identity and 62% similarity to ExsM. The main difference between ExsM and BxpC is in the C terminus, which is positively charged in ExsM but not in BxpC (93). BxpC was present in exosporium-deficient spores of an *exsB* mutant, suggesting that it may not actually reside on the exosporium, but perhaps in the interspace or outer spore coat (45).

CotE (BAS3619)

CotE is an outer spore coat protein that is required for anchoring of the exosporium layer to the spore coat. Mutants lacking CotE produce a normally appearing exosporium in strips that do not properly assemble around the spore (35). Released mature spores lack the exosporium layer, except for small fragments which remain adherent to the spore. Full-length CotE has not been detected in the exosporium layer, but a 12-kDa N-terminal fragment

of CotE was identified in the exosporia of *B. anthracis* and *B. cereus* (94). CotE of *B. subtilis* is a morphogenetic protein in spore coat assembly and affects outer spore coat assembly (95). Thus, its impact on exosporium attachment may be indirect, through defects in outer coat assembly. Henriques and Moran hypothesized that CotE may bridge the nascent coat to the exosporium during early exosporium synthesis and subsequently be cleaved to allow the exosporium to spatially separate from the coat to form the characteristic interspace (1).

CotY (BAS1145)

CotY was identified as an exosporium protein in a proteomic study of spores of *B. anthracis*, but it is also found in other members of the *B. cereus* family (34). The *cotY* gene maps to a cluster of exosporium determinants adjacent to the *bclA* locus (Fig. 7). CotY and ExsY are exosporium proteins that share substantial sequence similarity. Although their N-terminal sequences (~30 residues) are dissimilar, the rest of their sequences are highly conserved (~93% identical). The CotY protein localizes to the exosporium cap region (58).

ExsA (BAS4324)

Spores of an *exsA* mutant of *B. cereus* exhibited decreased hydrophobicity and increased permeability to lysozyme and were blocked at a late stage of germination (41). Truncation mutations resulted in defects in anchoring of the exosporium as well as outer spore coat layers. The location of ExsA within the spore has not been determined. Although it is named as a putative exosporium protein and involved in proper exosporium assembly, ExsA is not likely a true exosporium component. It possesses a conserved N-terminal cortex binding domain which suggests that it resides in the inner coat layer of the spore (41). ExsA displays sequence similarity to the *B. subtilis* SafA spore protein that functions in spore coat assembly. The *B. anthracis* Sterne protein is 85% identical to the *B. cereus* protein.

ExsB (BAS1898)

First identified in proteomic studies of *B. anthracis* and *B. cereus* exosporium proteins, ExsB is a polypeptide of unusual amino acid composition, with 20 of its 186 residues (in strain Sterne) being cysteine (45, 94). The protein is highly phosphorylated on threonine residues. Loss of ExsB results in unstable exosporium attachment, with loss of the exosporium layer in spores prepared in liquid medium and only fragments of exosporium present on spores prepared on solid medium (45). Aronson et al. (96) reported that this protein is located principally in the inner coat/cortex region of the spore, with little found in exosporium preparations. Thus, they named this protein Cotγ. The amount of Cotγ was found to be dependent on culture conditions (pH and temperature). Reduced levels of Cotγ resulted in spores lacking an exosporium or with an exosporium closely abutting the spore coat, and the resultant Cotγ-negative spores were less hydrophobic and germinated more rapidly than wild-type spores (96).

The *exsB* determinant has been shown to be transcribed by the σ^K-bearing form of RNA polymerase in *B. thuringiensis*, and its expression is additionally regulated by the GerE transcriptional factor (97).

ExsC (BAS2697)

ExsC was identified in a proteomic study of *B. anthracis* and *B. cereus* exosporium proteins (94). The ExsC protein of *B. cereus* migrates on SDS-PAGE gels at the size predicted for a dimer. The *B. anthracis* Sterne protein is 67% identical and 82% similar to the *B. cereus* protein.

ExsFB (BAS2303)

ExsFB is a protein with striking amino acid sequence similarity to ExsFA/BxpB. Mutants lacking ExsFB exhibit a slight reduction in BclA spore content (63). Double mutants lacking both BxpB and ExsFB exhibit a complete loss of the BclA nap layer and have an altered exosporium composition. The residual exosporium present in these double mutants is very fragile and is shed upon storage of the spores. Based upon fluorescent reporter localization studies, it was postulated that ExsFB may be found in the interspace region of the spore (35). My colleagues and I found that ExsFB initially is found at the cap pole of the spore (66). Levels of ExsFB were elevated in *bxpB*-null and *bclA*-null mutant spores, with the highest concentration at the cap and smaller amounts around the non-cap region of the exosporium.

ExsH

ExsH was identified as a collagen-like spore surface glycoprotein of *B. cereus* and *B. thuringiensis* (94, 98, 99). *B. cereus* ExsH has a C-terminal BclB C-terminal domain (exospore_TM), defined as a C-terminal region in a number of proteins that have extensive collagen-like triple helix repeat regions. The ExsH C-terminal BclB C-terminal domain is predicted by TmHMM (100) to have four transmembrane helices. The N terminus of this protein, upstream of the collagen-like repeats, is more extensive than those of BclA and BclB.

ExsJ

ExsJ was identified as a major glycoprotein in the *B. cereus* exosporium (94). ExsJ contains a collagen-like GXX repeat sequence and is 81% sequence identical to ExsH, with more of the variation at the N termini of these proteins, upstream of the collagen-like repeats. *B. cereus* ExsJ has a C-terminal BclB C-terminal domain (exospore_TM) predicted to have four transmembrane helices. The N-terminal domains of the ExsH and ExsJ proteins, upstream of the collagen-like repeats, are longer than those of BclA and BclB. ExsH and ExsJ exhibit some sequence similarity in the N-terminal domains, with 89 of the first 133 residues (67%) being identical.

ExsK (BAS2377)

ExsK was found to be present in at least two distinct locations within the *B. anthracis* spore, namely, on the surface beneath the BclA nap and in a more interior location, beneath the exosporium basal layer (101). ExsK is incorporated into the spore first at the mother cell proximal pole forming the cap and later becomes distributed around the rest of the spore. The amount of ExsK remains higher at the cap. However, in a mutant lacking the basal layer protein BxpB, ExsK localized to only one spore pole (101). In spores lacking BclA, ExsK fails to assemble into high-molecular-weight species as it does in wild-type spores. Mutant spores lacking ExsK were found to germinate faster and more extensively than their wild-type counterparts (101).

ExsM (BAS2174)

ExsM, a basic protein with a molecular mass of 15.4 kDa, was first identified in a proteomic study of *B. cereus* (94). *B. cereus exsM-*

null mutant spores were reported to be smaller and rounder than wild-type spores, and 77% of the mutant spores exhibited a double-exosporium arrangement, with both exosporia possessing the BclA nap layer (93). The ExsM-deficient spores were reported to be more resistant to lysozyme and germinated at a higher rate, but they exhibited a delayed outgrowth (93). Mutational loss of *B. anthracis exsM* produced less pronounced phenotypes with regard to spore size, yielded a lower percentage of duplicate exosporia (30%), and the exosporium was more fragile than that of the wild type (93).

ExsY (BAS1141)

ExsY is an exosporium protein that has been characterized in both *B. anthracis* and *B. cereus* (34, 42, 44). A *B. anthracis* Δ*exsY* mutant produces an exosporium only at the mother cell central pole of the spore (the cap), while the remainder of the exosporium fails to develop (42). This cap structure is lost from spores prepared in liquid medium but can be detected on spores prepared on solid medium. The cap site exosporium has a BclA nap but shows evidence of a disorganized basal layer. Boydston et al. (42) also reported that the exosporium sublayers (below the BxpB-containing basal layer) were more fragile and easily lost from the spores in the Δ*exsY* mutant. *B. cereus* spores (prepared in liquid medium) from a mutant strain containing an insertionally inactivated *exsY* determinant failed to produce an organized exosporium (44). The surfaces of the released spores were decorated with what appeared to be exosporium fragments (44).

GerQ (YwdL; BAS5242)

GerQ is an exosporium protein first characterized in *B. cereus* and *B. thuringiensis* (102, 103). Spores lacking GerQ produce a more fragile exosporium. Immunogold labeling revealed that GerQ is found on the inner surface of the exosporium. Mutant spores lacking GerQ had a reduced rate of germination in response to alanine plus inosine and failed to germinate in response to the germinant receptor-independent calcium dipicolinate (104). GerQ/YwdL is required for retention of CwlJ (a germination-specific cortex lytic enzyme) in the *B. subtilis* spore (104).

IunA (BAS4961)

IunA is a putative inosine-uridine-preferring nucleoside hydrolase identified as a *B. anthracis* exosporium protein (K. A. Spreng and G. C. Stewart, unpublished data). It is surface exposed but is more strongly recognized by anti-IunA antibodies in the absence of the BclA nap.

IunH (BAS2693)

IunH is an inosine-uridine-preferring nucleoside hydrolase first identified as a *B. anthracis* exosporium protein by Lai et al. (106) and Redmond et al. (34). The IunH protein, similar to inosine-uridine-preferring nucleoside hydrolases, is found in the spore, possibly in the interspace region (35).

Superoxide Dismutase Sod15 (BAS1378)

Superoxide dismutases were identified in exosporium preparations by Steichen et al. (33) and Liu et al. (74). Superoxide dismutase molecules within the spore may afford *B. anthracis* protection against oxidative stress and enhance pathogenicity in animal models of infection (107). The presence of multiple superoxide dismutase genes may provide redundancy needed to ensure protection of germinating spores in animal hosts. The *sod15* determinant is transcribed in the late exponential and sporulation phases, consistent with its spore localization (108).

Superoxide Dismutase SodA1 (BAS4177)

SodA1 was identified in exosporium preparations by Steichen et al. (33) and Liu et al. (74). Its contributions to oxidative stress and virulence were examined by Cybulski et al. (107). A strain lacking all four superoxide dismutases encoded in the *B. anthracis* genome was found to be attenuated for virulence upon intranasal challenge of mice (107).

Cataloging of exosporium proteins remains incomplete. My colleagues and I employed a genetic screen to identify genes whose proteins are important for proper incorporation of BclA into the exosporium (109). Several determinants were identified which either localize to the spore (based on gene fusion studies) or are required for full BclA assembly. These may represent either exosporium proteins or possibly nonspore proteins that participate in exosporium assembly.

FUNCTIONS OF THE EXOSPORIUM IN THE ENVIRONMENT

Spores present in soil environments constitute the source of animal infections by *B. anthracis* and other soil-associated pathogens. Furthermore, it has been shown that under the right conditions, *B. anthracis* spores can germinate on and around plant roots and that genetic exchange between strains of *Bacillus* is possible (110). Because of the spore mode of infection, it is important for spores to persist at their site of deposition and not to get washed too deeply into the soil and thus be inaccessible to grazing ruminants in amounts constituting an infectious dose (111). The exosporium affects the ability of *B. anthracis* spores to bind to different soil types. Wild-type spores of *B. anthracis* adhere more strongly than exosporium-deficient spores to soils with high organic material and calcium levels, i.e., soils which have been shown to favor the persistence of this pathogen (112). The presence of BclA on the surfaces of spores resulted in better retention of spores in a porous medium of silica sand (113).

The exosporium layer and the BclA glycoprotein, in spores possessing it, contribute to the overall hydrophobicity of the spore (80, 84, 99). The hydrophobic character contributes to the binding properties of the spore in the environment. Binding to food preparation surfaces is important to the foodborne pathogen *B. cereus*. Spore adherence to surfaces, such as stainless steel, contributes to the environmental persistence of the spores and ultimately to infection of foods by this bacterium.

Studies have attributed roles for the exosporium (114, 115) or BclA (78, 99) in binding to stainless steel surfaces. In contrast to the results obtained with *B. anthracis*, a study with a strain of *B. cereus* (ATCC 14579) indicated an increase in spore hydrophobicity in *bclA* deletion mutants, and this coincided with a reduction in spore binding to stainless steel (99). However, overall spore hydrophobicity does not adequately explain the differences in stainless steel adhesion properties. Other reports have concluded that the overall hydrophobicity of the spore and the presence of surface appendages by these spores have the greatest impacts on stainless steel binding (116–118).

FUNCTIONS OF THE EXOSPORIUM IN AN INFECTED HOST

The majority of studies concerned with spore infectious processes have focused on the zoonotic pathogen *B. anthracis*. This bacte-

rium has an unusual host-pathogen interaction. The bacterium, in order to disseminate as many infectious spores into the soil as possible, has evolved mechanisms to gain access to the host's bloodstream, where it replicates to large numbers and kills the host through elaboration of toxins. The sporulation process does not occur to any significant extent in the infected host until after the animal dies and postmortem carcass decomposition provides the oxygen-replete environment needed to induce sporulation. The large bacterial numbers then translate to large numbers of spores released into the soil around the decomposing carcass. To accomplish this infectious process, the infecting spores must transit from the site of infection to the draining lymph node, germinate to the vegetative bacterial form, and initiate invasion of the bloodstream and rapid replication. The exosporium, in particular the surface-exposed BclA protein, participates in many of the initial spore-host interactions.

Studies with animal models of disease and tissue culture-based infections have provided clues to the nature of the initial events in the infectious process. These studies indicate that when spores are introduced into an animal host, they are taken up by macrophages and dendritic cells (119–123). Spores in the lung are phagocytosed by alveolar macrophages or dendritic cells, which initiate the processes leading to pulmonary anthrax (120, 122, 123). For pulmonary infections, it has also been shown that *B. anthracis* spores are capable of entering epithelial cells of the lung and crossing this cell layer without apparent disruption of the barrier integrity (124, 125). The gastrointestinal form of anthrax ensues when spores are ingested and migrate to the intestinal tract, where they pass across the gut epithelial cell layer without germination. Once outside the intestines, the spores are taken up by the macrophages of the Peyer's patches (119). In either form of anthrax, the phagocytes migrate to the draining lymph nodes. The spores germinate *en route* within the macrophage or dendritic cell and begin to replicate and produce toxins. At the lymph node, the bacterium-containing phagocytes lyse, releasing the bacteria. Invasion of the bloodstream, replication, toxin elaboration, and death of the susceptible host ensue.

Uptake of spores by phagocytic host cells has been shown to involve interactions between BclA and the host integrin Mac-1 (CR3) (126). Spores lacking the BclA glycoprotein do not specifically target these cells but bind more generally to epithelial cells (127). CD14 was shown to bind to rhamnose residues of BclA and to act as a coreceptor for spore binding by Mac-1 (128). Oliva et al. proposed a model whereby binding of rhamnose to CD14 triggers signaling through Toll-like receptor 2 (TLR-2), leading to activation of phosphatidylinositide 3-kinases and converting Mac-1 into a more active receptor for *B. anthracis* spores (128). Survival studies using C57BL/6 and CD11b$^{-/-}$ mice revealed that CD11b$^{-/-}$ mice lacking Mac-1 were more resistant to infection with wild-type but not BclA-deficient spores (126, 128).

These results suggest that the spore surface BclA fibers may act to promote uptake by professional phagocytes and to inhibit nonspecific interactions between *B. anthracis* spores and nonprofessional phagocytic cells. Gu et al. reported that activation of the classical complement pathway is a primary mechanism of spore phagocytosis by murine macrophages (129). Phagocytic uptake of spores was found to be reduced significantly in the absence of complement component C1q or C3. C1q recruitment to the spore surface was shown to be mediated by BclA, which can directly bind C1q in a dose-dependent and saturable manner (129, 130). Fur-

thermore, C1q acted as a bridging molecule between BclA and integrin $\alpha_2\beta_1$ to mediate *B. anthracis* spore entry into epithelial cells in a complement activation-independent manner. A binding protein for the globular head domains of complement component C1q, designated gC1qR, was also reported to be involved in at least the initial stages of *B. cereus* spore attachment and/or entry (131).

The above findings suggest a central role for BclA in *B. anthracis* spore infection of hosts. However, loss of BclA has been shown to have no effect on virulence of *B. anthracis* in animal models of infection, as measured by 50% lethal dose (LD$_{50}$) values (84, 132). Additionally, the mean time to death following subcutaneous or intranasal inoculation of Δ*bclA* mutant spores in an A/J mouse model of infection was shorter than that with wild-type spores (84). Oliva et al. found that C57BL/6 mice were more susceptible to Δ*bclA* spores than to wild-type spores (the LD$_{50}$ of Δ*bclA* spores was approximately 5-fold lower than that of wild-type Sterne spores in a subcutaneous challenge model, and the mean time to death was shorter with the BclA-lacking spores) (126). They also reported similar results with A/J mice after intrathecal spore administration. A better understanding of the early events in the infectious process is needed before we can explain the discrepancy in results reported for animal disease models and why loss of the BclA spore surface glycoprotein either has a minimal impact on the lethal dose or creates an increase in virulence, depending on the animal model of infection utilized.

Spores of *B. anthracis* can persist in the lungs of infected hosts following aerosol exposure. For example, viable spores were found in the lungs of rhesus macaques at least 42 days following aerosol exposure (133–135) and in mouse lungs for more than 25 to 30 days (136, 137). Spores of *B. anthracis* were significantly better at persisting in the lung than spores of a non-exosporium-possessing *B. subtilis* strain, and some of the persisting spores were found within epithelial cells (138). Treatment of mice with an Src family kinase inhibitor significantly reduced *B. anthracis* dissemination from the lung to distal organs and prolonged the median survival time for mice compared to those for the untreated control group (139). There appears to be a signaling pathway specifically required for spore entry into epithelial cells, and these studies provided evidence suggesting that this pathway is important for dissemination and virulence *in vivo* (139).

Another possible mechanism whereby spores persist in the lungs involves innate immune system evasion. Interference with the innate immune system may initially occur upon introduction of spores into a susceptible host. Plasminogen efficiently binds to spores in an exosporium- and BclA-dependent manner (140). Plasminogen-bound spores are capable of exhibiting antiopsonic properties by cleaving C3b molecules, resulting in a decrease in macrophage phagocytosis, possibly contributing to a longer persistence of free spores in infected lung tissue.

Perhaps as a consequence of the decreased hydrophobicity of BclA-deficient spores, the extracellular matrix proteins laminin and fibronectin bound significantly better to Δ*bclA* mutant spores than to wild-type spores (84). BclA-negative spores were also found to adhere better to epithelial cells, fibroblasts, and endothelial cells but not macrophages. Spores lacking the exosporium layer were found to elicit a higher cytokine response (beta interferon, interleukin-1β [IL-1β], tumor necrosis factor alpha [TNF-α], and IL-6) in mouse macrophages than that in response to exosporium-bearing spores (141). This suggests a role for the exo-

sporium in avoidance of innate immune signaling during the early stages of spore infection.

Studies have suggested that the timing of spore germination within phagocytic cells is regulated to prevent premature germination of spores. The timing of germination is thought to be critical to the outcome of the spore-macrophage interaction (70). The exosporium is involved in the initial spore interactions with the intracellular compartment of phagocytes following spore uptake. Enzymes are present in the outer spore layers, apparently to prevent premature germination of the internalized spores and to provide protection to the emerging vegetative cell following germination of the spore (34). These spore-associated enzymes include alanine racemase to convert L-alanine (a germinant) to D-alanine (an inhibitor of L-alanine-induced germination) and at least two, and possibly more, inosine-preferring nucleoside hydrolases that may function to inactivate inosine (another germinant species) (35, 69, 70, 101, 142).

The *B. anthracis* exosporium contains an arginase that metabolizes L-arginine to L-ornithine and urea, thereby reducing the levels of arginine, the substrate for the host cell nitric oxide synthase (71, 72). This decreases the amount of NO radicals produced in spore-infected macrophages. Counterintuitively, *B. anthracis* spores have their own NO synthase. Spores lacking this enzyme were attenuated for virulence in an A/J mouse model of infection and were more susceptible to killing when germinating within macrophages (143). Resistance to macrophage killing is thought to be dependent on NO-mediated activation of bacterial catalase and suppression of the Fenton reaction. Additionally, a potential exosporium-associated virulence factor is superoxide dismutase, which may provide protection to the germinating spores against reactive oxygen species-mediated defense systems of phagocytic cells (107, 108). These spore-associated enzymes may protect the newly emerging vegetative cells at a time when they are very sensitive to killing by reactive oxygen species, until toxin production by the newly emerging vegetative bacteria can ensue and ultimately kill the host cell.

CONTRIBUTIONS OF EXOSPORIUM PROTEINS TO PROTECTIVE IMMUNITY

The first demonstration of an effective vaccine against anthrax in animals was conducted by Louis Pasteur at the famous public experiment at Pouilly-le-Fort, in May 1881, to demonstrate his concept of vaccination. The vaccine consisted of *B. anthracis* spores that were chemically attenuated for virulence. The anthrax vaccine widely used in veterinary medicine today is a live spore vaccine and involves the use of the Sterne strain, which lacks the pXO2 plasmid and thus cannot produce the bacterial cell capsule, an important virulence factor for this bacterium. This vaccine was originally developed at the Onderstepoort Laboratory, Pretoria, South Africa. Human vaccines against anthrax, in contrast, are cell-free preparations whose efficacy is considered to be due primarily to antibodies generated against the protective antigen component of the anthrax lethal and edema toxins. These include the American anthrax vaccine adsorbed (AVA; BioThrax) and the British anthrax vaccine precipitated (AVP). Animal studies of the efficacy of the human vaccine found that protection against the most virulent strains of *B. anthracis* required the addition of spore antigens to the vaccination regimen (144–147). Improved vaccine protection was observed when the added spore antigens included inactivated whole spores (147–150), live attenuated

spores (146, 151–154), BclA (81, 83, 148, 155), BxpB (68, 148), and BAS5303 (68, 148). Antibodies directed against whole spores or, specifically, BclA have been shown to be inhibitory to spore germination *in vitro* (148, 156). Studies involving passive immunization with spore-specific antisera have produced mixed results. Protection has been reported in some studies but could not be demonstrated in others (148, 157–159). The distinct difference between the unambiguous benefit obtained with active immunization with PA and spore components and the less clear-cut results obtained via passive immunization involving anti-spore-component antibodies underscores the likely involvement of cellular immunity in protection (159).

BACTERIA WITH EXOSPORIUM-CONTAINING SPORES

Several endospore-producing bacteria have been reported to possess exosporia. Examples are listed in Table 1. In addition, genes similar to *bclA* of *B. anthracis* are present in the genome sequences of *Clostridium autoethanogenum* DSM 10061, *Clostridium beijerinckii* NCIMB 8052, *Clostridium ljungdahlii* DSM 13528, *Clostridium saccharolyticum* WM1, and *Clostridium ramosum*.

Bacillus megaterium

There appears to be strain heterogeneity with regard to exosporium production in *B. megaterium*. An exosporium was visible in spores of more than half of 36 strains examined (163). The *B. megaterium* exosporium was described in detail and a hairlike nap layer seen on the spore surface in an electron microscopy study by Beaman et al. (181). Hexagonal periodicity was observed with the exosporium basal layer. In addition, longer fibrillar material with a laminar appearance radiated outward from the spore surface. Planar inclusions were evident in the interspace region when spores were visualized by transmission electron microscopy. A novel morphogenetic spore coat protein, BMQ_0737, was found to be important for the proper assembly of the outer spore coat and exosporium layers of the *B. megaterium* QM B1551 spores (182). A pole-localized nap layer of strain QM B1551 is composed of the BclA1 protein, and this polar nap localization is independent of BxpB (183). A plasmid-free derivative of strain QM B1551 was found to lack the exosporium layer, and the spores were less hydrophobic (183). Of the seven resident plasmids, only pBM600 and possibly pBM500 are required for exosporium production in sporulating cells of this strain. Plasmid-carried orthologues of *B. cereus* family exosporium genes were identified. Two *bclA*-like determinants are carried on pBM500 and were designated *bclA1* and *bclA2*. An orthologue of the *B. cereus* family BxpB/ExsFA exosporium basal layer protein is encoded on plasmid pBM600. Fluorescent protein fusion studies indicated that the BclA1 protein localizes around the developing forespore, but fluorescence was not detected on the surfaces of released spores (183).

Clostridium botulinum

In an early study, spores of *C. botulinum* type E were found to possess a fragile exosporium layer with tubular appendages (with various lengths of up to 0.56 μm and a 200-Å diameter, with a lumen of 80 Å; a hemispherical cap composed of 40-Å spherical subunits is present at the end of the appendage) present in the interspace region of the spore (171). In a later report involving a type A strain, spores were reported to possess exosporia which were multilayered in appearance, containing up to 15 layers (172).

TABLE 1 Exosporium-producing bacteria

Organism	Comment	Reference(s)
Bacillus anthracis		160, 161
Bacillus badius	Spore surface appendages present	161, 162
Bacillus cereus	Spore surface appendages present	61
Bacillus circulans	Spore surface appendages present	162
Bacillus firmus	Spore surface appendages present	161
Bacillus fusiformis		162
Bacillus lentus	Spore surface appendages present	161, 162
Bacillus megaterium	Strain-variable feature	163
Bacillus mycoides		162, 164, 165
Bacillus niacini		162
Bacillus pseudomycoides		162
Bacillus pumilus		162
Bacillus thiaminolyticus	Spore surface appendages present	161
Bacillus thuringiensis	Spore surface appendages present	166
Bacillus vedderi		162
Brevibacillus brevis	Spore surface appendages present	161
Brevibacillus laterosporus		162, 167
Clostridium bifermentans	Spore surface appendages present	168
Clostridium botulinum	Tubular surface appendages	168–172
	Multilayered exosporium basal layer	
	Has nap; interspace layered filaments	
Clostridium difficile	Strain-variable feature?	173, 174
	No prominent interspace with most strains (perhaps a crust layer?)	
Clostridium pasteurianum	Exosporium incompletely envelopes the spore (has a terminal opening)	175
Clostridium septicum	Multilayered	176
Clostridium sordellii	Spore surface appendages present	168
Clostridium sporogenes	Spore surface appendages present	168
Lysinibacillus odysseyi		162, 177
Lysinibacillus sphaericus	The insecticidal binary toxin is located in the spore interspace adherent to the internal surface of the exosporium	178, 179
Paenibacillus alvei	Spore surface appendages present	162
Pasteuria penetrans	Peripheral fibers for spore attachment within the interspace	180
Rummeliibacillus pycnus		162
Viridibacillus neidei	Spore surface appendages present	162

The layers were uniform in appearance and were 3 nm thick, with a center-to-center distance of 7 nm.

Clostridium difficile

Several articles have been published that refer to an exosporium layer of *C. difficile* (recently proposed to be reclassified as *Peptoclostridium difficile*) (184). In only one publication is there electron microscopic evidence of a possible traditional exosporium layer with a defined basal layer and interspace region (185). In this report, as in others, micrographs indicate an external crust-like layer (such as that shown in Fig. 8) or fragments of material adherent to the spore coat in strains of *C. difficile* (13, 174, 185, 186). Joshi et al. also indicated a pronounced variation in spore hydrophobicity among strains, with the more hydrophobic strains possessing a more organized exosporium (173). These studies suggest that possession of an exosporium may not be a feature of *C. difficile* spores or may be a strain-variable trait in this bacterium. An alternative possibility is that the exosporium layer is extremely fragile and readily lost from spores (13). The more likely possibility is that *C. difficile* spores lack a true exosporium layer (as proposed previously [13]) and possess a crust layer, albeit one with a pronounced hairlike nap. The bacterium exhibits strain variability in the appearance of the crust layer nap. A proteomic study did not reveal exosporium proteins similar to the known *B. cereus* family
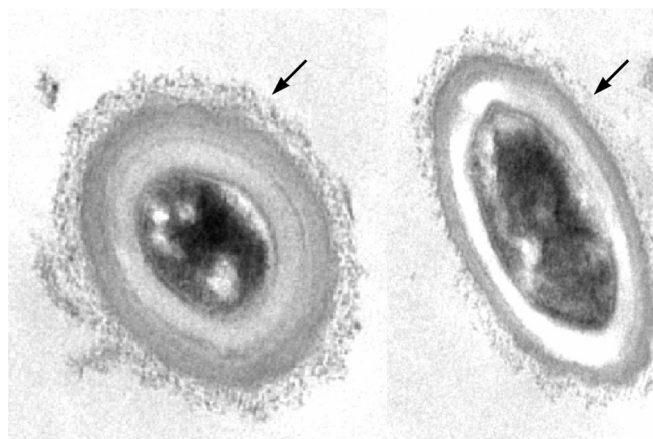

FIG 8 Transmission electron micrographs of *C. difficile* strain ATCC 700057 spores. The spores were stained with ruthenium red, a carbohydrate stain to identify the glycoprotein layer of the spore surface. The spores feature a prominent cortex region (the white area surrounding the core). An interspace region, the area between the exosporium and the outer spore coat, is not an obvious feature in *C. difficile* spores. The ruthenium red-stained putative glycoprotein layer is denoted by arrows.

exosporium proteins, except for the presence of collagen-like proteins (187). However, the lack of *B. cereus* family exosporium homologs does not rule out the existence of *C. difficile* exosporium structures, since genomic analyses of known exosporium producers outside the *B. cereus* family suggest that exosporium proteins differ markedly in various species.

Three genes encoding collagen-like glycoproteins have been identified in the *C. difficile* genome (*bclA1*, *bclA2*, and *bclA3*), and the encoded proteins are expressed during sporulation and localize to the spore, with BclA1 likely present in the outermost "exosporium/crust" layer of the spore (13, 188). BclA1, unlike BclA of *B. anthracis*, was reported to be poorly immunogenic in this study. ClosTron-generated mutants of the *bclA1* and *bclA2* determinants produced spores with altered surfaces, including release of "sheets of coat-like material" from the spores (188). BclA3 was identified as a glycosylated protein in extracts of *C. difficile* spores, and the glycosyltransferase gene *sgtA*, carried upstream of *bclA3*, is at least partly responsible for glycosylation of the spore surface (189).

A cysteine-rich protein (CdeC) localized to the outermost spore layer was found to be important for the formation of the nap layer of the *C. difficile* spore and might be the anchoring protein linking the outermost layer to the spore coat (190). The CotA protein was shown to be important for proper assembly of the outermost spore layer, and the CotA, CotE (a bifunctional peroxiredoxin reductase and chitinase), CotF, and SodA (superoxide dismutase) proteins were found to be associated with the spore surface (185). The *C. difficile bclA1*, *bclA2*, *bclA3*, *cdeC*, *cotA*, *cotCB*, *cotD*, and *cotE* genes were determined to be dependent on σ^K for expression; this is the same sigma factor involved in exosporium gene expression in the *B. cereus* family of organisms (191).

A proteomic analysis of proteins extracted from the outermost layers of *C. difficile* strain 630 identified 184 distinct proteins (192). Proteins were extracted from spores by use of trypsin treatment or sonication methods. In this report, Flag fusion studies indicated an "exosporium" location for the BclA1, BclA2, BclA3, CdeA, CdeB, CdeC, and CdeM proteins.

The outermost layer of *C. difficile* spores has been reported to interact with a receptor or receptors on intestinal epithelial cells (193) and may have an adverse effect on macrophage phagosomal membranes (194). Proteolytic treatment of spores decreased adherence to the cells. Removal of the outermost spore layer(s) was also reported to reduce adherence to colonic cells, decrease overall spore hydrophobicity, and increase spore colony-forming frequencies *in vitro* (13, 173). The more pronounced exosporium-like layers may correlate with increased spore hydrophobicity and better adherence to gut epithelial cell monolayers and environmental stainless steel surfaces (174). Loss of each of the BclA1–3 glycoproteins resulted in decreased spore hydrophobicity. Relative to the wild-type parent strain, mutant spores lacking BclA1 were shown to have a 2-log higher infectious dose requirement in a mouse model of infection and displayed reduced virulence in a hamster infection model (188). BclA1 is thought to be important in the initial colonization of spores in the gut epithelium of the infected host.

Clostridium sporogenes

Spores of the anaerobic bacterium *C. sporogenes* possess an unusual exosporium feature. The exosporium is reported to have a protrusion, or "sporiduct," at one pole of the spore, which in some spores becomes an opening or aperture in the exosporium (195,

196). Functions ascribed to the exosporium include roles in germination, outgrowth, and attachment (195, 197). A recent report indicates that the germination and outgrowth process for *C. sporogenes* strain ATCC 15579 involves rupture of the spore coat at a site adjacent to the exosporium aperture (196). The emerging cell exits the spore coat and exosporium through these aligned openings.

Lysinibacillus sphaericus (formerly Bacillus sphaericus)

A lamellar exosporium with a hexagonal arrangement of subunits was reported in an electron microscopic study of sporulation in the mosquitocidal bacterium *L. sphaericus* (178). Within the spore interspace, the insecticidal parasporal crystal is enclosed in a mesh-like parasporal envelope (198). A collagen-like protein, BclS, was identified as being part of filamentous structures in the interspace region of the spores (199). Heat resistance and germination defects were noted in spores lacking BclS.

Pasteuria penetrans

Pasteuria penetrans is a parasite of root-knot nematodes whose spores are the infectious form. The spores have been commercialized as a biological control agent (200). *Pasteuria* endospores have additional structures within the interspace. The perisporium largely consists of peripheral fibers that are present in the interspace and attached to the spore body. These fibers are involved in attachment of the spores to the nematode cuticle (180). After attachment to host cuticle cells, the exosporium is lost, exposing the peripheral fibers to the cuticles of nematode juveniles. Binding of spores to the host cuticle has been proposed to involve collagen-like fibers on the surface of the endospore interacting with mucins on the nematode cuticle (201).

Pasteuria ramosa

P. ramosa infects water fleas of the *Daphnia* genus. Infected hosts are completely sterilized and have a reduced life span. A collagen-like protein containing GXY repeats (Pcl1a [*Pasteuria* collagen-like protein 1a]), one of 37 collagen-like proteins encoded in the *P. ramosa* genome, was identified in a proteomic analysis of spore surface proteins of *P. ramosa* (202, 203). The specific location of the Pcl1a protein in the spore has not been determined.

THE EXOSPORIUM AS A SPORE DISPLAY PLATFORM

The BclA collagen-like glycoprotein is abundantly expressed on the surfaces of spores of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. The N-terminal 35 amino acids of BclA have been shown to be sufficient for localization of the protein around the spore and its stable incorporation onto the exosporium basal layer surface (57, 65). It is possible to display foreign proteins on the surfaces of *B. anthracis*, *B. cereus*, or *B. thuringiensis* spores by incorporating the 35 BclA N-terminal codons at the N-terminal end of the foreign gene open reading frame and expressing it in sporulating cells by utilizing the *bclA* promoter and ribosome binding site. The foreign protein is synthesized by the sporulating cells and stably incorporated in large numbers at the spore surface. The recombinant protein-decorated spores are easily purified and can be employed as microparticles for industrial, bioremediation, or vaccine applications (H. Y. Hsieh, G. C. Stewart, B. M. Thompson, and C. H. Lin, unpublished data). Enzymes that retain activity when tethered at the N terminus are ideal for these biocatalytic processes. The spore display system is ideally suited for soil environ-

ments, where spores can remain in place following application, whereas free enzymes tend to wash out and are more readily inactivated.

CONCLUSIONS

Our understanding of the composition, assembly process, and function of the spore exosporium layer remains incomplete. The presence of exosporium proteins in large SDS-resistant complexes hampers studies of specific protein-protein interactions. As with other aspects of endospore formation, genetic studies will likely be the best route to a more complete understanding of the composition, assembly pathway, and function of this outermost spore layer. The distribution of sugar residues on the spore surface glycoproteins and the roles that they may play are understudied aspects of spore biology. Atomic force microscopy was recently utilized to begin to define the distribution of sugars on the surfaces of *B. cereus* spores (205). Regulation of exosporium formation warrants further investigation. Recently, it was discovered that production of spores of a particular strain of *B. cereus* under conditions of anaerobiosis resulted in defects in the exosporium structure (206). Based on what we know regarding spore formation by *B. anthracis*, it could be anticipated that reduced oxygen tension might result in reduced sporulation efficiencies. However, structural defects in the exosporia of the formed spores were unexpected. How exosporia evolved in different species by using unique building blocks will be a fascinating field of study as our understanding of non-*B. cereus* group exosporium composition improves. It is apparent that the studies begun in the 19th century by Robert Koch still have a lot to disclose regarding the biology of endospores.

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